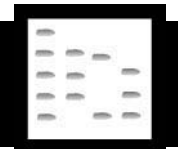
IN-DEPTH DISTRIBUTION IN QUANTITATIVE TLC



I. Vovk and M. Prošek, National Institute of Chemistry, Ljubljana, Slovenia

Copyright © 2000 Academic Press

Among many techniques used for quantification of thin-layer chromatograms, slit-scanning densitometry is the most common. It gives the opportunity to choose between either reflectance or transmission mode according to the nature of the supporting material. For instance, glass-backed plates can only be used for transmission measurements above 320 nm due to absorption of the UV radiation, while aluminium-backed plates are opaque at all wavelengths.

The matrix of sample and stationary phase, which consists of small particles, is optically opaque and strongly light-scattering. Therefore, densitometric measurements of separated substances are much more difficult than equivalent photometric measurements in solution. A further problem with densitometry is the fact that it is unable to detect the vertical and radial concentration profiles of the separated substance within the spot as a result of diffusion and the chromatographic process.

Reflectance and transmission are particularly sensitive to changes in sorbent quality, thickness, spot shape and size, eluent and development conditions. To reduce the errors due to plate-to-plate variation, standards should always be applied to the same plate as the unknown samples. The errors due to migration differences as a result of edge effects, deviations in layer thickness and nonlinear solvent fronts can be further minimized using the data-pair technique introduced by Bethke and co-workers in 1974. This technique is based on an internal compensation, by pairing up the measurements of two spots on the same plate.

Most thin-layer chromatography (TLC) analysis is performed in the UV and visible spectral region by applying reflectance scanning densitometry. However, the results of reflectance measurements are restricted to the surface of the sorbent on the TLC plate and are therefore strongly dependent upon the in-depth distribution of the analysed compound inside the sorbent. Nonuniformity of the in-depth distribution of a compound inside the sorbent as a result of secondary chromatography can occur during the evaporation–drying stage. Differences in the in-depth concentration profiles of the samples and standards can cause significant errors and cannot be completely eliminated by the data-pair technique.

Theoretical Problems of Optical Scanning Densitometry

All optical methods for the quantitative evaluation of planar chromatograms are based on measuring the difference in optical response between blank regions of the stationary phase and regions with a separated substance. When monochromatic light falls on an opaque medium, some light may be reflected from the surface, some may be absorbed by the medium and converted to heat, and the remainder is diffusely reflected or transmitted by the medium. Regularly (specularly) reflected light does not give any useful information of the sample distributed within the sorbent, however, it can contribute to the noise signal in scanning densitometry as it cannot be distinguished from the diffusely reflected light. Quantification in TLC is based on measuring the diffusely reflected or transmitted light and assuming that the specularly reflected component from the sorbent is very small.

The propagation of light within an opaque medium is a very complex process that can be mathematically solved only by assuming certain simplifications. The transmission and reflectance of light in highly scattering media has been discussed by Chandrasekhar (1950) in his book on radiative transfer, where he gives the basic integro-differential radiation transport equation. As this equation has no analytical solution, all useful equations and theories have been developed by simplifying the actual case.

Continuum theories of absorption in opaque media, such as Kubelka-Munk's theory, are not appropriate for quantitative TLC. Although they describe the absorption and scattering properties of the medium, they do not take into account the interaction of light with individual particles in the layer and the very important problem of nonuniform in-depth distribution of a compound within the sorbent. Bodo, Johnson and Melamed have developed well-known discontinuum theories for the determination of absolute optical constants from the properties of individual sample particles. In 1968 Goldman and Godal published their paper on the theoretical basis of measurements by optical transmission and reflection in silica gel layers using a modified

DropBooks

Kubelka-Munk equation. The theoretical background of *in situ* evaluation of thin-layer chromatograms was described by Kortüm in 1969.

Theoretical studies of the problems in light scattering are important in order to reach an understanding of the measuring principle and to help the analyst to find more precise methods of evaluating the data. However, one of the main difficulties is the fact that the adsorbed substance is never homogeneously distributed over the whole sorbent which is required in theory for a relationship between reflectance and concentration of the adsorbed substance.

Multilayer Models

The effect of the concentration gradient of the substance on the intensity of the signal in reflectance and transmission measurements has been studied by a number of authors. Most have studied the theoretical aspect of this problem. Nevertheless, using different approaches, they have all come to the same conclusion. They showed that densitometric transmittance measurements and fluorescence measurements from the far (nonilluminated) side of a plate yield results which are almost independent of the in-depth distribution of the analysed material. In contrast, the densitometric reflectance and fluorescence measurements from the near (illuminated) side are strongly dependent on that distribution. They suggested using transmittance or fluorescence transmittance mode whenever there are reasons to suspect a nonhomogeneous distribution of concentration or a changing coefficient of fluorescence of the separated material in the depth of the chromatogram.

However, as already pointed out, most densitometric measurements are preformed in reflectance mode, and are therefore restricted only to the surface of the sorbent. It is for this reason that differences in the in-depth distribution of compounds (samples and standards) can cause erroneous results. Prošek and his co-workers have investigated the effect of the concentration gradient on the intensity of the diffusely transmitted and reflected light. They solved multilayer models using the mathematical theory of Bodo and Markov chains. In such a model a chromatographic band was placed in different sublayers (**Figure 1**). Although their equations do not offer any improvement from a practical point of view, they show the effect of the concentration gradient in the sorbent layer that the Kubelka-Munk theory cannot. The practical application of their theory was confirmed using the real models prepared from different kinds of layers (paper and TLC sorbents). The results obtained from these real models were in good agreement, confirming theoretical

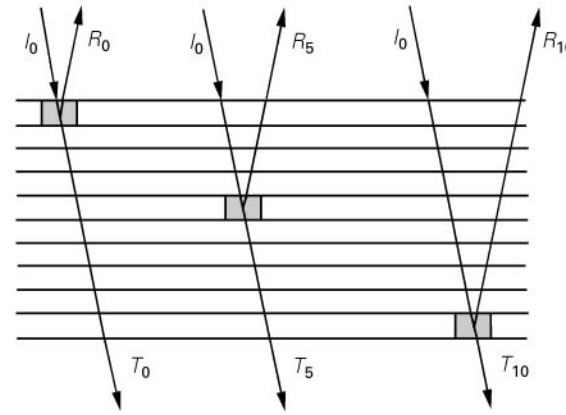


Figure 1 Schematic presentation of a multilayer model. *I*, incident beam; *R*, reflected beam; *T*, transmitted beam.

results and showing that reflectance densitometric measurements do not take into account the vertical subsurface concentration distribution.

Further investigations have been made with image analysing systems, an emerging detection technique in TLC. In the case of image analysing systems, higher radiation fluxes must be taken into account as the result of the illumination of a whole plate. Compared to slit-scanning densitometry, the intensity of diffuse light inside the illuminated layer is much higher, as higher numbers of reflected beams come from all parts of the layer. The results obtained by measuring multilayer models with a charge coupled device (CCD) camera (**Figure 2**) showed the effect of the position of the spot inside the layer on the signal. As can be seen from **Figure 3**, the intensity of the signal in the reflectance measurements is highly dependent on the position of the spot inside the layer. This has confirmed that differences in the in-depth distributions of standards and samples can cause significant errors in reflectance quantitative TLC. On the other hand, the position of the spot inside the layer has almost no effect on the signal in transmittance measurements.

Secondary Chromatography

While drying a TLC plate, molecules of compounds are moving with the evaporating mobile phase in the direction of the sorbent's surface. This process is called secondary chromatography and results in inhomogeneous vertical distribution of compounds inside the sorbent. This effect is bigger for compounds having high R_F values in the solvent being evaporated.

Variations of in-depth distribution have a big impact on densitometric reflectance measurements. This

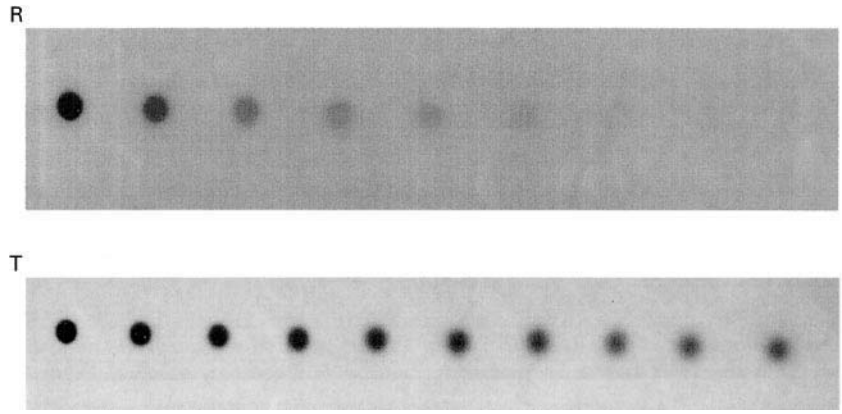


Figure 2 CCD images of a 10-layer model while measuring reflectance (R) and transmission (T) with a Camag video documentation system.

is one of the reasons for the nonlinearity of calibration curves and may lead to the erroneous interpretation of TLC chromatograms by reflectance densitometry. It is therefore very important to find drying conditions which will give TLC plates with the most homogeneous and consistent depth distribution of compounds.

Depth Profiling of TLC Plates by Photoacoustic Spectroscopy

Preliminary investigations of nondestructive depth profiling of TLC plates have been performed. The results obtained using different photothermal techniques, showed that photoacoustic spectroscopy (PAS) is most suitable for characterization of TLC plates. Although PAS has been used previously for the qualitative and quantitative spectroscopic analysis of TLC plates, the first results of photoacoustic depth profiling of TLC plates have only recently been published.

PAS is a photothermal technique, which relies on the detection of pressure waves (sound) generated by the absorption of radiation in a periodically irradiated sample. It has the capability for *in situ* and nondestructive depth profiling of solid samples. This unique feature is due to the fact that the magnitude of the induced photothermal effect depends on the concentration and on the thermal diffusivity of a compound. The plot of the dependence of the photoacoustic (PA) signal on the modulation frequency provides information about the depth profile of the analysed compound.

DropoBooks

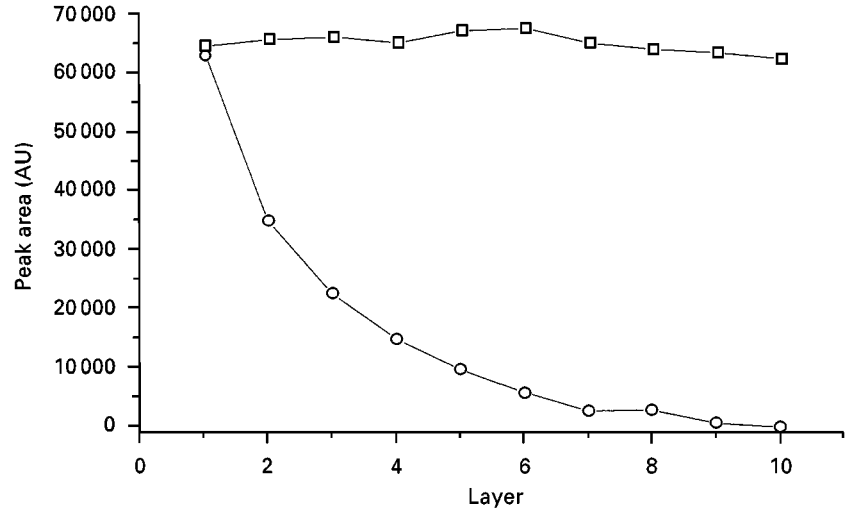


Figure 3 The effect of the spot position in the 10-layer model on the reflectance (circles) and transmission (squares) measurements with a Camag video documentation system.

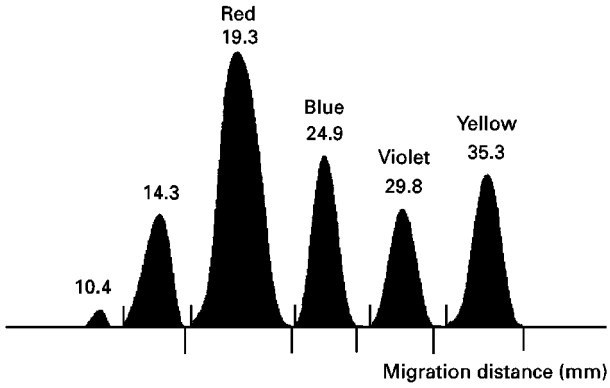


Figure 4 Densitogram obtained from Camag test dye mixture III. (Reproduced with permission from Vovk I, Franko M, Gibkes J, Prošek M and Bicanic D (1997) Depth profiling of TLC plates by photoacoustic spectroscopy. *Journal of Planar Chromatography* 10: 258. Copyright Research Institute for Medicinal Plants in cooperation with Springer Hungarica.)

The PA signals were analysed using the theory for a two-layer model consisting of the sorbent and the glass as the supporting material. Thermal diffusivity values (α) of the spots on TLC plates were obtained by curve fitting of normalized phase lags of PA signals. Different thicknesses of sample, corresponding to the thermal diffusion length μ, given as $\mu = \sqrt{(\alpha/\pi f)}$, were probed by varying the frequency (f) of the laser beam modulation.

The PA signals originating from different layers of a TLC plate were calculated by subtracting the values obtained at higher modulation frequencies from those obtained at lower frequencies. From these two modulation frequencies and the previously obtained thermal diffusivities of each spot, the depth and thickness of each layer can be determined. Depending on the available range of modulation frequencies used in the PA measurements, the thickness of the probed layers varied from 23 to 37 μm. All the results were corrected for differences in layer thickness.

In-Depth Distribution of Compounds Inside the Sorbent and Quantitative TLC

The effect of nonhomogeneous in-depth distribution of compounds on quantitative TLC has been studied on TLC and high performance TLC (HPTLC) plates using the separation of a test dye mixture (**Figure 4**). The results of PA investigations showed that all compounds exhibited nonhomogeneous concentration profiles in a vertical direction. Although all the investigated compounds tended to concentrate in the upper 25% of a 250 μm thick layer, secondary chromatography can lead to deviations from this behaviour. Additionally, differences in the in-depth concentration distribution of different compounds on the same TLC plate were seen (**Figures 5** and **6**). While the yellow dye spots concentrated in the top 31 μm layer, the highest concentration in the violet spots was observed in the region between 39 and 61 μm. This indicates that the effects of secondary chromatography depend strongly on the properties of the compounds in each spot. The situation is different in the case of HPTLC plates, where

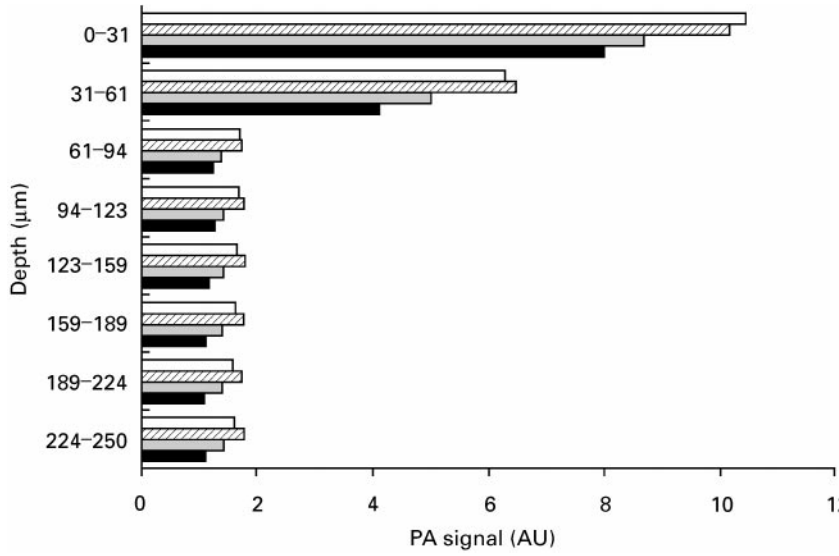


Figure 5 Depth distribution of the compound in yellow spots of equal concentration. Open column, track no. 4; cross-hatched column, track no. 3; dotted column, track no. 2; filled column, track no. 1. (Reproduced with permission from Vovk I, Franko M, Gibkes J, Prošek M and Bicanic D (1997) Photoacoustic investigations of secondary chromatographic effects on TLC plates. *Analytical Science* 13 (Suppl.): 191. Copyright The Japan Society for Analytical Chemistry.)

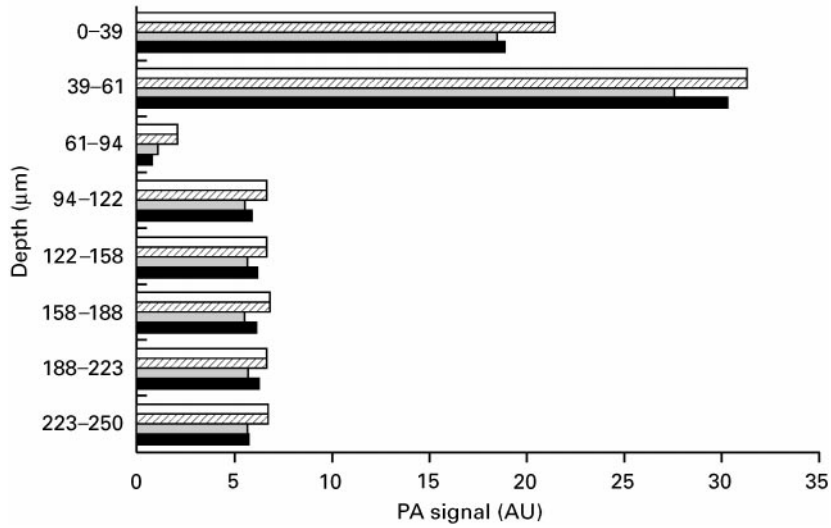


Figure 6 Depth distribution of the compound in violet spots of equal concentration. Open column, track no. 4; cross-hatched column, track no. 3; dotted column, track no. 2; filled column, track no. 1. (Reproduced with permission from Vovk I, Franko M, Gibkes J, Prošek M and Bicanic D (1997) Photoacoustic investigations of secondary chromatographic effects on TLC plates. *Analytical Science* 13 (Suppl.): 191. Copyright The Japan Society for Analytical Chemistry.)

even the violet spots tends to concentrate in the upper, 0–37 μm layer (**Figure 7**). Different in-depth distribution of compound inside the sorbent of TLC and HPTLC plates can be explained by 50 μm differences in the layer thickness. In the case of the thinner layer of the HPTLC plate, the evaporation of the mobile phase is faster and causes faster movement of the substance to the surface of the plate. Differences in PA signals from the same depths were observed for spots from different tracks. This indicated that nonuniformity within one TLC plate could be the source of erroneous quantification in scanning densitometry (Figures 5–7). Additionally, when monitoring only the surface of the TLC plate, by reflectance densitometry, such irregular vertical concentration distribution can result in the nonlinearity of calibration curves (**Figure 8**). For the same reasons a nonlinear calibration curve is obtained by PAS, when the probed sorbent layer is too thin (top curve in **Figure 9**). Taking into account all the con-

DropBooks

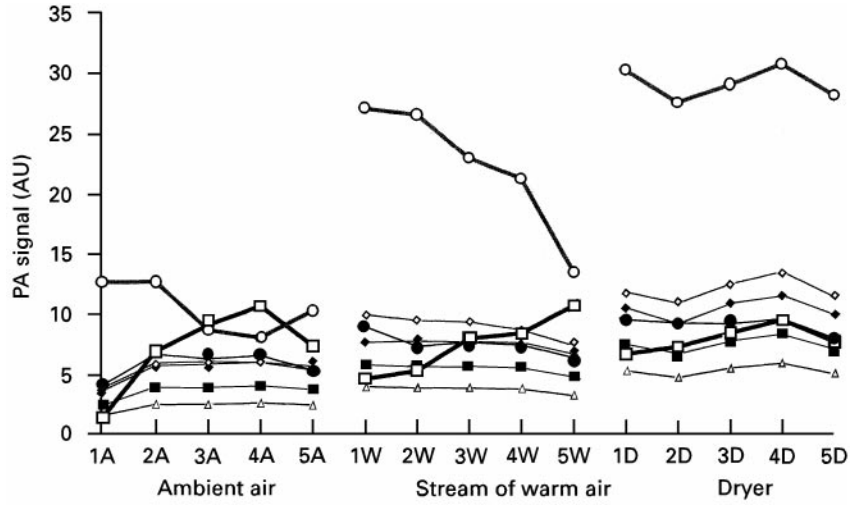


Figure 7 Depth distribution of the compound in violet spots on HPTLC plates dried in ambient air (A), in a stream of warm air (W) or in a dryer (D). Open circles, 0–37 μm; open squares, 37–62 μm; filled circles, 62–88 μm; open diamonds, 88–125 μm; open triangles, 125–148 μm; filled squares, 148–176 μm; filled diamonds, 176–200 μm. (Reproduced with permission from Vovk I, Franko M, Gibkes J, Prošek M and Bicanic D (1998) The effect of drying conditions on the in-depth distribution of compounds on TLC plates investigated by photoacoustic spectroscopy. *Journal of Chromatography* 11: 379. Copyright Research Institute for Medicinal Plants in cooperation with Springer Hungarica.)

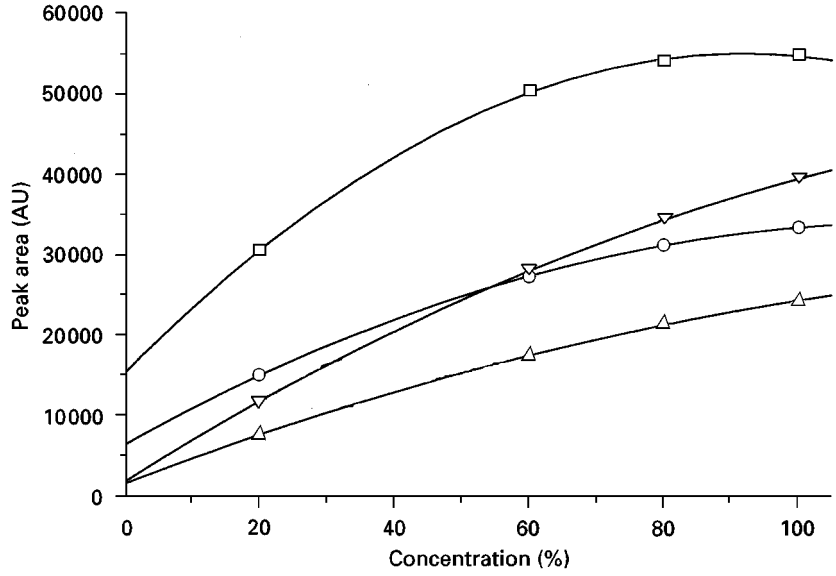


Figure 8 Calibration curves for the Camag test dye mixture III (squares, red spots; circles, blue spots; triangles, violet spots; inverted triangles, yellow spots) obtained by reflectance densitometry. (Reproduced with permission from Vovk I, Franko M, Gibkes J, Prošek M and Bicanic D (1997) Photoacoustic investigations of secondary chromatographic effects on TLC plates. *Analytical Science* 13 (Suppl.): 191. Copyright The Japan Society for Analytical Chemistry.)

siderable irregularities in vertical concentration distribution (61 μm) by PA probing of thicker sorbent layers (lower curves in Figure 9) leads to improved linearity of calibration curves compared to those obtained by reflectance densitometry.

Our investigations confirmed that in TLC not only accurate application, development and quantitative evaluation, but also accurate drying are essential to obtain good results. Unfortunately, up to now the importance of this procedure has not been fully appreciated. The effects of drying process on the in-depth distribution of compounds inside the sorbent on HPTLC plates has therefore been investigated for drying in a dryer, in a stream of warm air

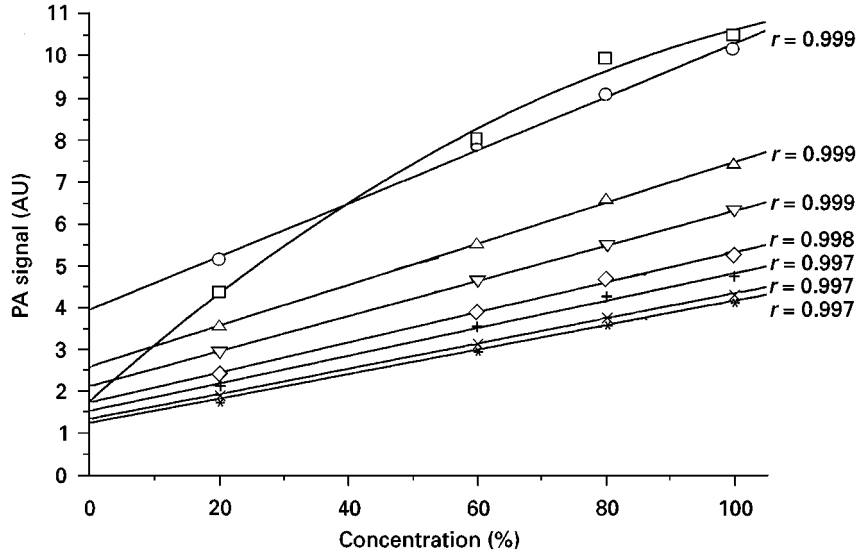


Figure 9 Calibration curves for the yellow spots obtained by PAS when probing different thicknesses of the sorbent. Squares, 0–36 μm; circles, 0–61 μm; triangles, 0–94 μm; inverted triangles, 0–123 μm; diamonds, 0–159 μm; crosses, 0–185 μm; multiplication signs, 0–225 μm; asterisks, 0–250 μm. (Reproduced with permission from Vovk I, Franko M, Gibkes J, Prošek M and Bicanic D (1997) Photoacoustic investigations of secondary chromatographic effects on TLC plates. *Analytical Science* 13 (Suppl.): 191. Copyright The Japan Society for Analytical Chemistry.)

Table 1 Peak areas obtained by densitometric measurements of violet spots on three different HPTLC plates, dried in the ambient air (A) or in a stream of warm air (W) or in a dryer (D)

	Peak areas (a.u.) for violet spots						
	Track 1	*Track 2*	*Track 3*	*Track 4*	*Track 5*	*Mean*	*RSD (%)*
Ambient air	39 037	38 992	38 876	36 908	36 250	38 012.6	3.50
Warm air	38 242	38 886	38 788	37 889	35 311	37 823.2	3.86
Dryer	36 707	36 089	36 857	36 445	36 755	36 570.6	0.84

Reproduced with permission from Vovk I, Franko M, Gibkes J, Prošek M and Bicanic D (1998) The effect of drying conditions on the in-depth distribution of compounds on TLC plates investigated by photoacoustic spectroscopy. *Journal of Planar Chromatography* 11: 379. Copyright Research Institute for Medicinal Plants in cooperation with Springer Hungarica.

and in ambient air. The results obtained by PAS studies from different HPTLC plates were compared to those obtained by reflectance densitometry.

A comparison of the response areas was obtained for five violet dye spots from HPTLC plates dried in ambient air (A), in a stream of warm air (W) and in a dryer (D). The highest relative standard deviation (RSD) values were obtained for the plate dried in a stream of warm air (**Table 1**). The RSD for drying in a dryer was significantly lower than the RSD values for drying in the ambient air or in the stream of warm air. This can be explained by the nonuniform drying conditions across the HPTLC plate while drying in a stream of warm air.

The results of PA measurements are presented in Figure 7. For most of the tracks on all three HPTLC plates, the highest PA signals were detected in the top 37 μm thick layer. Significant differences in PA signals were observed under different drying conditions in the top 62 μm layer of the sorbent. Differences in the in-depth distribution of compounds due to variations of drying conditions across the same HPTLC plate are especially remarkable when the results for all five tracks from one plate (A, W or D) are compared (Figure 7). The largest variation was observed between tracks on the HPTLC plate dried in the stream of warm air. The most reasonable explanation for the large differences in PA signals observed between the plates dried in ambient air and those dried in a dryer or in the stream of warm air is the difference in radial distribution of the compound within the spots. When a TLC plate is dried in ambient air, the compound is radially distributed over a larger area as a result of diffusion. In the case of drying in the dryer and drying in a stream of warm air, the spots are shrinking due to migration of molecules in a radial direction towards the centre of the spot. This results in an increased concentration, and therefore higher PA signals (Figure 7), when the radius of the laser beam is smaller than the spot radius, as was the case. In the case of densitometric measurements the probed area is larger than the diameter of a spot so that radial changes in concentration cannot be observed.

The magnitude of secondary chromatography, and consequently the vertical as well as the radial concentration distribution of compounds in the sorbent, depends on drying conditions. It also depends on the properties of the analytes and the type of TLC plate (TLC or HPTLC). Drying in a dryer gives the most reproducible results, while drying in a stream of warm air gives the least reproducible results.

DropBooks

Future Trends

We anticipate that in-depth distribution will have less effect on quantitative TLC in the future, because developments in the field of TLC plates is focused on producing thinner layers by using stationary phases with a smaller and more defined particle size. However, until this is achieved, further studies of secondary chromatography are necessary.

In addition, the wider use of image-analysing systems, mostly working in the visible part of the spectrum, will increase the number of methods using postchromatographic derivatization and quenching techniques. These procedures should also be tested by PAS to elucidate their effect on the in-depth and radial distribution of compounds on TLC plates.

See also: **II/Chromatography: Thin-Layer (Planar):** Densitometry and Image Analysis; Instrumentation.

Further Reading

Bein K and Pelzl J (1989) In: Analysis of surface exposed to plasmas by nondestructive photoacoustic and photothermal techniques. Auciello O and Flamm DL (eds) *Plasma Diagnostics*: *Surface Analysis and Interactions*, p. 211. New York: Academic Press.

Gibkes J, Vovk I, Bolte J *et al*. (1997) Photothermal characterisation of TLC plates. *Journal of Chromatography A* 786: 163.

Prošek M and Pukl M (1996) Basic principles of optical quantitation in TLC. In: Sherma J and Fried B (eds) *Handbook of Thin-layer Chromatography*, p. 273. New York: Marcel Dekker.

Prošek M, Medja A, Kučan E *et al*. (1979) Quantitative evaluation of thin-layer chromatograms: The calculation of remission and transmission using multilayer model. *Journal of High Resolution Chromatography & Chromatographic Communications* 2: 517.

Prošek M, Medja A, Kučan E *et al*. (1980) Quantitative evaluation of thin-layer chromatograms: The calculation of fluorescence using multilayer models. *Journal of High Resolution Chromatography & Chromatographic Communications* 3: 183.

Vovk I, Franko M, Gibkes J *et al*. (1997) Depth profiling of TLC plates by photoacoustic spectroscopy. *Journal of Planar Chromatography* 10: 258.

Vovk I, Franko M, Gibkes J *et al*. (1997) Photoacoustic investigations of secondary chromatographic effects on TLC plates. *Analytical Science* 13(suppl): 191.

Vovk I, Franko M, Gibkes J *et al*. (1998) The effect of drying conditions on the in-depth distribution of compounds on TLC plates investigated by photoacoustic spectroscopy. *Journal of Planar Chromatography* 11: 379.

INDUSTRIAL ANALYTICAL APPLICATIONS: SUPERCRITICAL FLUID EXTRACTION

M. E. P. McNally, DuPont de Nemours, Wilmington, DE, USA

Copyright © 2000 Academic Press

By the mid-1980s, routine analysis was accomplished in most industrial laboratories with the help of automated sampling and data-handling systems. The next major bottleneck caused by the burden of large sample loads that existed in many industrial environments was sample preparation and the method development time associated with it. Robots and automated systems were introduced to address these bottlenecks. Supercritical fluid extraction (SFE) was one of the automated systems introduced.

The initial introduction of SFE was unique, in that supercritical fluid chromatography (SFC) was developing at a parallel pace. Both introductions into the analytical community produced scientists who needed to learn the nuances of a totally new fluid, its capabilities, limitations, likeness to and difference from both gases and liquids.

SFE was embraced more readily in the industrial community than SFC. The reasons for this, in its utility as well as its developments, are considered in more detail in this overview.

Actual industrial applications of any analytical technology are sometimes well-guarded secrets. References can come from a variety of sources, not necessarily the published literature. Trends in the applications of SFE are no different. The most use has been in the food industry and the environmental area. Additional applications to a lesser extent are in polymer analysis, consumer products and pharmaceuticals.

Beyond the ability to conduct an analysis previously unachievable, the driving force for the adaptation of a technique into the industrial laboratory depends on a financial benefit. SFE has solved some problems that could not previously be solved. But it has mostly gained an industrial foothold by offering worthwhile cost savings over the already acceptable methodology, such as liquid extraction.

Instrumental Developments

In the development of SFE, industrial applications were the driving force to the advancement of commercial instrumentation. Initial offerings of commercial equipment involved cumbersome sample vessels with a myriad of problems. Not uncommonly, these were placed in single-vessel extraction units. At most, the instrumentation was capable of conducting SFC following the supercritical extraction. Speeds of analysis were typically measured by actual extraction time, and not overall sample handling, operator time or productivity. In this early equipment, productivity was not truly improved and industrial acceptance was slow. This equipment was only viable for those extraction methods that could not be conducted by another extraction solvent, i.e. a liquid or a gas. This led SFC, where both liquid and gas chromatography had a strong hold in the separation field, to be labelled as a niche technique. In the sample preparation field, where alternative automated extraction methods with liquids and gases were not as firmly